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Homotropic allosteric control in clostridial glutamate dehydrogenase: Different mechanisms for glutamate and NAD^+?

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Abstract **Clostridial glutamate dehydrogenase mutants with the 5 Trp residues in turn replaced by Phe showed the importance of Trp 64 and 449 in cooperativity with glutamate at pH 9. These mutants are examined here for their behaviour with NAD^+ at pH 7.0 and 9.0. The wild-type enzyme displays negative NAD^+ cooperativity at both pH values. At pH 7.0 W243F gives Michaelis–Menten kinetics, and the same behaviour is shown by W243F and also W310F at pH 9.0, but not by W64F or W449F. W243 and W310 are apparently much more important than W64 and W449 for the coenzyme negative cooperativity, implying that different conformational transitions are involved in cooperativity with the coenzyme and with glutamate.**

Keywords: Glutamate dehydrogenase; Negative cooperativity; Allosteric mechanism; Trp/Phe mutants; Homotropic interaction; Site-directed mutagenesis; Hill coefficients

1. Introduction

The family of hexameric glutamate dehydrogenases includes a number of members displaying striking allosteric characteristics [1–3]. Among the best known and kinetically elaborate is the enzyme from bovine liver, selected by Monod et al. [4] to exemplify the allosteric concept in 1963. This enzyme shows both homotropic responses to its own reaction coenzymes [5] and also heterotropic responses to such mononucleotides as GTP [3,4]. It is also now clear that a distinctive structure, the 'antenna', serves as a heterotropic allosteric site [6]. This structure is contributed by a C-terminal portion of about 50 amino acid residues that are missing from the sequences of many of the homologous glutamate dehydrogenases (NAD^+-dependent) EC 1.4.1.2 (GDHs) of prokaryotes and simpler eukaryotes [1,2,7,8].

It is accordingly important to note that these shorter GDHs still offer several well-documented examples of allosteric behaviour [7,9,10]. In the absence of one layer of regulatory complexity, these simpler GDHs may offer a route to understanding the fundamental structural basis of inter-subunit communication. Among these enzymes, the NAD^+-dependent GDH of *Clostridium symbiosum* has both a structure solved at high resolution [11] and a detailed kinetic description [12]. The previous kinetic studies have revealed two separate manifestations of homotropic allosteric effects under distinctly different conditions. First of all, at neutral pH, whilst the dependence on glutamate concentration appears to follow the conventional Michaelis–Menten pattern, the behaviour with NAD^+ is complex and shows a pattern of apparent negative cooperativity [12] rather reminiscent of that described many years ago for both NAD^+ and $NADP^+$ with the bovine enzyme [3,5]. On the other hand, at pH 8.8–9.0, a pattern initially described as a pH-dependent inactivation [13], was later shown to be an aspect of an allosteric conformational equilibrium [14] that could be reversed (reactivation) by glutamate with a remarkable degree of positive cooperativity reflected in a Hill coefficient very close to the theoretical maximum of 6 for a hexamer [15]. For purely historical reasons, the behaviour with variation of coenzyme concentration has not previously been examined in detail at high pH.

Since these two homotropic responses were observed under different conditions, there has been no basis for assuming a direct link in terms of either the conformational states or the physical mechanism responsible for inter-subunit communication. Mutational studies have revealed that each effect can persist in the absence of the other when binding sites are disabled (e.g. [16,17]), but this does not unambiguously indicate whether the two effects rely on separate structural machinery. Recently, however, pursuit of Trp residues thought to be sensing the pH/glutamate-dependent conformational change [14], has highlighted two, W64 and W449, which appear to provide six pairwise interactions across the trimer–trimer interface [18]. Replacement of either of these Trp residues with Phe results in an enzyme that still undergoes 'inactivation' at high pH, but now the response to glutamate shows a greatly diminished Hill coefficient of about 2 [18]. It was therefore of interest to explore the behaviour of such mutants in their response to varied coenzyme concentration.

2. Materials and methods

2.1. Materials

Grade II NAD^+ (free acid) was obtained from Roche (Germany) and L-glutamate (monosodium salt), from Sigma Chemical Co. Sepharose CL-6B was from Pharmacia Biotech and IPTG from Melford (UK). Other routine chemicals and reagents were also of analytical grade. Remazol Brilliant Red GG was obtained from DyStar Textilfarben GmbH (Germany).

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Abbreviations: GDH, glutamate dehydrogenase (NAD^+-dependent) EC 1.4.1.2; *h*, Hill coefficient

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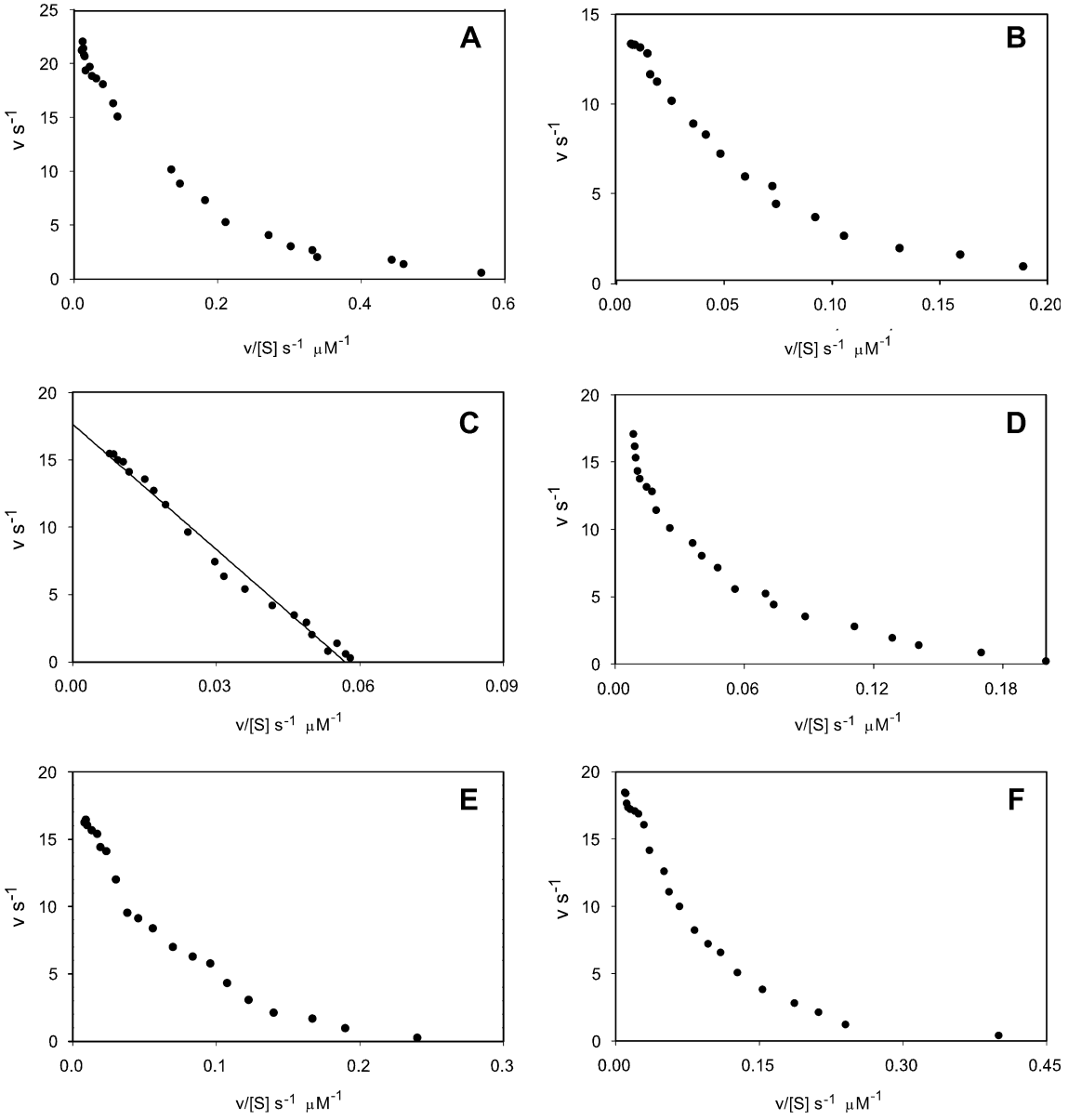

Fig. 1. Eadie–Hofstee plots for wild-type clostridial glutamate dehydrogenase and Trp/Phe single mutants in 0.1 M phosphate buffer at pH 7.0. Initial rates of reaction in the oxidative deamination direction were measured over a widely varied range of NAD^+ concentration (1–2000 μM) and with a fixed concentration of 40 mM glutamate by following the change in the fluorescence due to NADH production using a Hitachi fluorimeter. The readings were taken at λ_{Ex} 340 and λ_{Em} 560, and the fluorimeter was calibrated using a tetraphenyl butadiene cell as a reference fluorescent material. Each of the main panels (A–F) shows an Eadie–Hofstee plot of the initial-rate data. A is wild-type CsGDH; B is W64F; C is W243F; D is W310F; E is W393F; F is W449F.

Table 1
Hill coefficients for the NAD^+ concentration dependence of the rate of reaction catalysed by clostridial glutamate dehydrogenase and a series of Trp/Phe single mutants

Enzyme	pH 7.0		pH 9.0	
	Hill coefficient	V_{max}	Hill coefficient	V_{max}
WT	0.78 ± 0.025	23.71 ± 0.47	0.75 ± 0.089	35.16 ± 2.31
W64F	0.84 ± 0.048	15.13 ± 0.53	0.66 ± 0.12	41.79 ± 12.89
W243F	1.00 ± 0.040	18.36 ± 0.50	1.01± 0.10	19.72 ± 1.07
W310F	0.62 ± 0.040	24.89 ± 2.20	1.15 ± 0.20	34.25 ± 1.91
W393F	0.77 ± 0.039	19.63 ± 0.66	0.82 ± 0.14	14.48 ± 0.47
W449F	0.88 ± 0.065	20.97 ± 0.76	0.87 ± 0.30	17.63 ± 2.80

Initial-rate data over an NAD^+ concentration range from 1 to 2000 μM were fitted to the Hill equation using nonlinear regression and SigmaPlot software. In each case the Hill coefficient and standard deviation are shown together with the corresponding V_{max} value also with its standard deviation.

2.2. Bacterial strains and vectors

Escherichia coli TG-1[(*lacIPq* ^{TM}M15-pro). *SupE, thi, hsdD5/F', traD36, proA+B+)*], used as a host strain for all plasmids, was grown routinely at 37 °C in Luria–Bertani medium supplemented with ampicillin 100μg/ml, unless otherwise stated. Wild-type and mutant GDHs were all expressed using the plasmid expression vector ptac 85 [19], as previously described [20].

2.3. Enzyme preparation

The wild-type and mutant proteins [18] were purified as described by Syed et al. [12] on Remazol-Red columns from extracts of cell pastes of overnight-grown cell cultures of *E. coli* TG1 harbouring the corresponding gene [20]. Enzyme preparations, judged to be pure by both SDS–PAGE and measurements of specific activity, were stored as ammonium sulphate precipitates as described elsewhere [18]. Aliquots of the stored enzymes were prepared for use by dialysing against at least 1000 volumes of 100 mM potassium phosphate buffer, pH 7.0, or 50 mM bicine buffer, pH 9.0, centrifuging and filtering through 0.22 μm syringe-top filters. The concentration of each enzyme was determined spectrophotometrically by measuring the absorbance at 280 nm using the method of Gill and von Hippel [21].

2.4. Enzyme assays

Initial reaction rates for the oxidative deamination reaction were measured fluormetrically on a Hitachi 4500 fluorimeter with the concentration of NAD^+ systematically varied over a wide range, 1–2000 μM. In the first set of experiments, the fixed concentration of glutamate was 40 mM in 0.1 M potassium phosphate buffer, pH 7, in one series, and 400 mM in the other, in 50 mM bicine buffer, pH 9.0. The enzymes used in each set of experiments were dialysed against the corresponding buffer, as mentioned above. Filtered reaction mixtures (0.22 μm acrodisc syringe filters) were incubated at 25 °C for 10 min

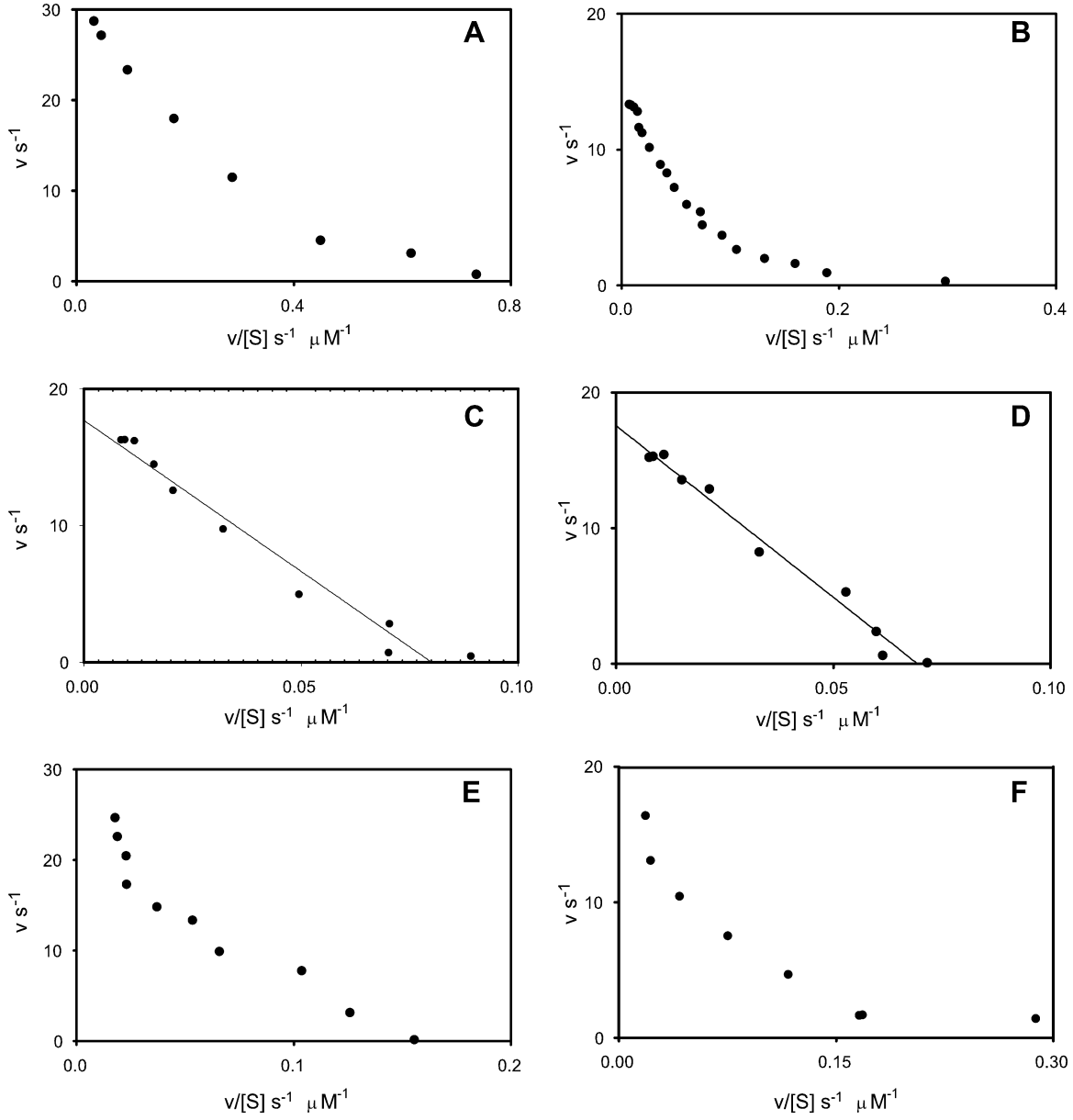

Fig. 2. Eadie–Hofstee plots for wild-type clostridial glutamate dehydrogenase and Trp/Phe single mutants in 50 mM bicine buffer at pH 9.0. Initial rates of the reactions in the oxidative deamination direction were measured by varying the NAD^+ concentration over a wide range (1–2000 μM) and with a fixed concentration of 400 mM glutamate, following the change in the fluorescence using a Hitachi fluorimeter adjusted as explained in Fig. 1. Each of the main panels (A–F) shows an Eadie–Hofstee plot of the initial-rate data. A is wild-type CsGDH; B is W64F; C is W243F; D is W310F; E is W393F; F is W449F.

prior to addition of enzyme. Initial-rate data were plotted as Eadie–Hofstee plots, making use of the Enzpack software package (Oxford Biosoft, UK). The same data sets were also fitted by nonlinear regression to the Hill equation by using SigmaPlot (Systat Software Inc., USA).

3. Results and discussion

It has been noted elsewhere [18] that the specific activities of the five Trp/Phe mutants measured at pH 7 and with high concentrations of both glutamate and NAD^+ are remarkably similar. In keeping with this observation, the initial-rate data in Fig. 1A–E show that the activities measured at high NAD^+ concentrations converge on very similar values, ranging from $13.3 \pm 0.4\ s^{-1}$ for W64F to $21.2 \pm 0.5\ s^{-1}$ for WT with 2 mM NAD^+. At neutral pH, the rates measured at low concentrations, on the other hand, show much larger differences, ranging from $0.05\ s^{-1}$ with W243F to $0.56\ s^{-1}$ with WT. Inspection of the graphical plots of these data (Fig. 1) reveals that, like the WT CS GDH, all the mutants, with the exception of W243F, show a marked pattern of negative cooperativity. Even in the case of W243F, it is arguable that the Eadie–Hofstee plot is faintly biphasic, but it is possible to fit the data, as shown, with a single straight line with relatively small deviation. This mutant, if not showing perfect Michaelis–Menten behaviour, comes very close to it, in marked contrast to all the other mutants and the wild-type enzyme. Correspondingly computer fits of these data to the Hill equation by nonlinear regression (Table 1) give values of the Hill coefficient (h) <1 for WT GDH and all of the mutants with the exception of W243F, for which h is 1.0. The most marked negative cooperativity is seen in W310F with an h value of 0.62.

At pH 9.0, where the detailed dependence on coenzyme concentration has not previously been studied, wild-type GDH showed a similar, remarkable pattern of negative cooperativity (Fig. 2A) with a Hill coefficient (0.75) very close to that determined at pH 7 (0.78) (Table 1). Three of the mutants similarly showed negative cooperativity, the exceptions being W243F and W310F (with Hill coefficients of 1.01 and 1.15). One can therefore draw two clear conclusions. First, replacement of W243 in the active site pocket by Phe virtually abolishes the strong pattern of negative cooperativity at both pH values. Replacement of W310 only has this effect at the higher pH. The involvement of W243 and W310 is not surprising since these two residues are in the active site cleft and, more specifically, in the vicinity of the coenzyme binding site. Second, although the subunit interface residues, W64 and W449, appear to play a crucial role [18] in mediating the positively cooperative response to glutamate at pH 9, their replacement with Phe has relatively little effect on the behaviour with NAD^+at pH 7.0 or 9.0. Although there are changes in the h values (Table 1), negative cooperativity is retained for both these mutants at both pH values.

It should be noted that, whilst in W64F and W449F the communication between trimers appears to be interrupted [18], these mutants do still display some more limited positive glutamate cooperativity, presumably restricted to interaction within the three subunits of each trimer. It is not clear whether the negative cooperativity in response to NAD^+ requires interaction between all six subunits of the hexamer. If not, it could still be argued that a single conformational transition in the trimer might account for both manifestations of cooperativity, i.e. with glutamate and with NAD^+, and that this is merely enhanced in the case of glutamate at pH 9 by a further level of interaction between trimers. An attractive option, therefore, would be to examine the behaviour of the enzyme under conditions where it forms trimers rather than hexamers. We have previously approached this in two ways. First of all, although it has been suggested that mild denaturing conditions produce active trimers [22], our own investigation with both urea [23] and guanidinium hydrochloride (S.A. Aghajanian and P.C. Engel, unpublished data) established clearly that there is no significant population of intermediate oligomers between the hexamer and monomer state. Secondly, a single site-directed mutation to destabilise the trimer–trimer interface resulted in an equilibrium distribution in which the hexamer was the only active species [24]. The desired experiment may, therefore, be unattainable. At this stage, however, the simplest explanation for the results reported in this paper is that the two allosteric effects described for this enzyme involve different conformational transitions. The pH/glutamate-dependent transition is between an active and an inactive conformation, whereas the coenzyme appears to mediate a transition between different active states and presumably therefore also involves different structural machinery.

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